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PATENT

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of: Sharat Singh  
Serial No.: Not Yet Assigned  
Filed: December 15, 1999  
For: Individually Addressable Surfaces For  
Multiplexed Operations

) Examiner: Not Yet Assigned  
)  
) Art Unit: Not Yet Assigned  
)  
) **UTILITY PATENT APPLICATION  
TRANSMITTAL**  
)

**BOX PATENT APPLICATION**

Commissioner of Patents and Trademarks  
Washington, D.C. 20231

Sir:

This is a request for filing a patent application under 37 C.F.R. § 1.53(b) in the name  
of inventor: **SHARAT SINGH**.

For: Individually Addressable Surfaces For Multiplexed Operations

This application is a [ ] Continuation [ ] Divisional  Continuation-in-part of  
prior Application No.: 60/113,853, filed December 24, 1998 from which priority under  
35 U.S.C. § 120 is claimed.

**Application Elements:**

20 Pages of Specification, Claims and Abstract  
4 Sheets of formal Drawings

**CERTIFICATE OF EXPRESS MAILING**

"Express Mail" Label No.: EL398977958US  
Date of Deposit: 15 December 1999

I hereby certify under 37 C.F.R. 1.10 that this correspondence is  
being deposited with the United States Postal Service as "Express  
Mail Post Office to Addressee" with sufficient postage on the date  
indicated above and is addressed to the Commissioner of Patents  
and Trademarks, Washington, D.C. 20231.

Chinde S. Matsumi  
(Signature) Chinde S. Matsumi  
(Printed Name)

Declaration

Unexecuted Combined Inventor Declaration and Power of Attorney  
 Copy from prior application (37 CFR 1.63(d) for a continuation or divisional).

The entire disclosure of the prior application from which a copy of the declaration is herein supplied is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

Deletion of inventors Signed statement attached deleting inventor(s) named in the prior application, *see* CFR 1.63(d)(2) and 1.33(b).

Accompanying Application Parts:

Assignment and Assignment Recordation Cover Sheet (recording fee of \$40.00 enclosed)  
 Power of Attorney  
 37 CFR 3.73(b) Statement by Assignee  
 Information Disclosure Statement with Form 1449  
 Copies of IDS Citations  
 Preliminary Amendment  
 Return Receipt Postcard  
 Small Entity Statement(s)  Statement filed in prior application.  
Status still proper and desired.  
 Other:  
 A sequence listing.  
 paper copy.  
 computer readable copy.  
 Statement in Compliance with Requirements for Patent Applications Containing Nucleotide and/or Amino Acid Sequence.

Claim For Foreign Priority

Priority of \_\_\_\_\_ Application No. \_\_\_\_\_ filed on \_\_\_\_\_  
is claimed under 35 U.S.C. § 119  
 The certified copy has been filed in prior application U.S. Application No. \_\_\_\_\_  
 the certified copy will follow.

Extension of Time for Prior Pending Application

A Petition for Extension of Time is being concurrently filed in the prior pending application. A copy of the Petition for Extension of Time is attached.

Amendments

- Amend the specification by inserting before the first line the sentence: "This is a  
 Continuation  Continuation-in-part  Divisional application of copending prior
- Application No. 60/113,853 filed on December 24, 1998.
- International Application \_\_\_\_\_ filed on \_\_\_\_\_, which  
designated the United States, disclosure of which is incorporated herein by reference."
- Cancel in this application original claims \_\_\_\_\_ of the prior application before  
calculating the filing fee

Fee Calculation (37 CFR § 1.16):

<u>FOR:</u>	<u>NO. FILED</u>	<u>NO. EXTRA</u>	<u>SMALL ENTITY</u>		<u>OTHER THAN A SMALL ENTITY</u>	
			<u>RATE</u>	<u>Fee</u>	<u>RATE</u>	<u>Fee</u>
Basic Fee			\$380	\$380	\$760	\$380
Total Claims	18	0	\$ 9	\$0	\$ 18	\$
Indep Claims	4	1	\$ 39	\$39	\$ 39	\$39
<input type="checkbox"/> Multiple Dependent Claims			\$130	\$0	\$260	\$
Total Filing Fee:						\$419

**TOTAL FEES: \$419.00**

- A check including the amount of the above-indicated TOTAL FEES is attached.
- Please charge Deposit Account No.18-0020 in the amount of \$ \_\_\_\_\_.
- No fee is required.
- Conditional Petition for Extension of Time: An extension of time is requested in the present and/or the above-referenced parent application to provide for timely filing if an extension of time is still required after all papers filed with this transmittal have been considered.
- The Commissioner is hereby authorized to charge any underpayment of the following fees associated with this communication, including any necessary fees for extension of time, or credit any overpayment to Deposit Account No. 18-0020.

- Any filing fees under 37 CFR 1.16 including fees for the presentation of extra claims.
- Any parent application processing fees under 37 CFR 1.17.
- A **duplicate** copy of this sheet is attached for accounting purposes.

Respectfully submitted,

Dated: December 15, 1999

By:

Bertram I. Rowland

Bertram I. Rowland, Ph.D.

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BRV/jsz  
Enclosures

Applicant: Sharat Singh

Patentee \_\_\_\_\_

Application No. \_\_\_\_\_ Patent No. \_\_\_\_\_

Filed on \_\_\_\_\_ Issued on \_\_\_\_\_

**COPY**

Title: Methods for Polynucleotide Analysis

**VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS  
(37 C.F.R. 1.9(f) and 1.27(c))-SMALL BUSINESS CONCERN**

I hereby declare that I am

the owner of the small business concern identified below.  
 an official of the small business concern empowered to act on behalf of the concern identified below.

Name of Small Business Concern: ACLARA BioSciences, Inc.

Address of Small Business Concern: 3906 Trust Way, Hayward, CA 94545-3716

I hereby declare that the above identified small business concern qualifies as a small business concern, as defined in 13 C.F.R. 121.12, and reproduced in 37 C.F.R. 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office under Sections 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to, and remain with, the small business concern identified above, with regard to the invention described in

the specification filed herewith, with title as listed above.  
 the application identified above.  
 the patent identified above.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. 1.9(c), if that person made the invention, or by any concern which would not qualify as a small business concern under 37 C.F.R. 1.9(d) or a nonprofit organization under 37 C.F.R. 1.9(e).

\*NOTE. Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. 1.27)

Each such person, concern or organization having any rights in the invention is listed below:

No such person, concern, or organization exists.  
 Each such person, concern or organization is listed below.

Name \_\_\_\_\_

Address \_\_\_\_\_

INDIVIDUAL       SMALL BUSINESS CONCERN       NONPROFIT ORGANIZATION

Name \_\_\_\_\_

Address \_\_\_\_\_

INDIVIDUAL       SMALL BUSINESS CONCERN       NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 C.F.R. 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1 001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Herbert H. Hooper

Title of Person if Other Than Owner: Executive Vice President

Address of Person Signing: 3906 Trust Way, Hayward, CA 94545-3716

SIGNATURE Herbert H. Date 12/23/72

APPLICATION  
FOR  
UNITED STATES LETTERS PATENT

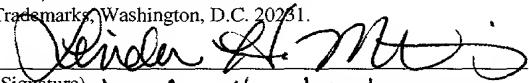
**TITLE: INDIVIDUALLY ADDRESSABLE SOLID  
SURFACES FOR MULTIPLEXED OPERATIONS**

**INVENTOR: SHARAT SINGH**

Certificate of Express Mailing

Express Mail Label Number: EL399977958 US  
Date of Deposit: 15 December 1999

I hereby certify Under 37 C.F.R. 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above, and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20261.

  
(Signature) Linda H. Matsunaga  
(Printed Name)

**INDIVIDUALLY ADDRESSABLE SOLID SURFACES FOR MULTIPLEXED  
OPERATIONS**

**CROSS-REFERENCE TO RELATED APPLICATIONS**

5

This application is a continuation-in-part of provisional application serial no. 60/113,853, filed December 24, 1998, which disclosure is incorporated herein by reference.

10

**INTRODUCTION**

**BACKGROUND**

There is increasing interest in the ability to identify nucleic acid sequences, nucleotide differences from nucleic acid sequences from different sources, 15 identification of polymorphisms, mutations and chiasmas, as well as other nucleic acid related analyses. As an increasing number of genomes from different sources are being subjected to sequencing, the ability to compare sequences has ramifications in identifying evolutionary development of species and families and identifying relationships between species. Also, by identifying fossil and progenitor DNA one 20 can estimate time periods in the development of clades, which can be of interest to archaeologists, geologists, anthropologists, etc.

In view of the large number of nucleotides in mammalian and plant genomes, when comparing genomes it will generally be desirable to make a plurality of determinations in a single assay. The ability to perform a number of simultaneous 25 events in a single reaction vessel with a complex DNA sample is frequently limited by erroneous events, where sequences may mismatch, there may be errors in replicating a DNA template, inherent errors in detection and the like. Besides DNA there is frequently an interest in being able to detect, desirably in a quantitative or semi- 30 quantitative manner the level of transcription of the genes in a cellular host. Since many of the mRNAs are in low ratios to other mRNAs, techniques must be devised to be able to identify the presence of the mRNAs in lesser amounts.

Microarrays have been developed to identify sequences in genomes. However, the microarrays have many deficiencies. Their fabrication is complex and very expensive. For each new group of nucleic acid segments to be assayed, a new microarray has to be prepared. In addition, they are subject to mismatching giving 5 false positives. Simpler, more economical and flexible methods are needed.

There is also interest in being able to identify proteins, particularly two or more proteins that complex together to form an active assembly, which has physiological significance. Where one has a complex mixture of proteins, it is of interest to determine, which proteins in the mixture form complexes and how other 10 proteins may affect such complex formation.

## RELEVANT ART

U.S. Patent no. 5,565,324 and Still et al., Accounts of Chem.Res., 1996, 29:155 describe a combinatorial approach to the synthesis of small organic molecules 15 using halocarbons on particles as the identifiers of the product, which halocarbons are detected by gas chromatography with flame ionization detection. U.S. Patent no. 5,578,498 describes metal chelate containing compositions for use in chemiluminescent assays.

20

## SUMMARY OF THE INVENTION

Methodologies and compositions are provided using individually addressable solid moieties, particularly lanthanide coded particles in liquid arrays, where the moieties whose fluorescent dyes are imbided into the moieties include one or more 25 fluorescent dyes, at least one being a chelated lanthanide fluorescent dye, for individual coding at individual sites, where the sites may be individual particles. The solid moieties, particularly particles, are prepared in kits for use in detecting a plurality of events occurring in the same assay mixture, where the dye labeled solid moieties may be bound with various agents, particularly nucleic acid sequences, at the 30 surface of the moieties. The agents may be covalently conjugated to the individual solid surface. The solid moieties may be used in the determination of the presence of

specific sequences in nucleic acid samples, the transcription of mRNA in cells, the presence of single nucleotide polymorphisms (snp's), the presence of mutations or other distinctive base or sequence of bases in a genome, the interaction between proteins and other entities, and the like. Various protocols may be employed with the liquid arrays and various detection schemes may be employed for deconvoluting the plurality of solid moieties resulting from the operation.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

10 Figure 1 is a schematic of an assay utilizing dethiobiotin-biotin release, where detection of a single target mRNA is realized on a microfluidic chip. Background is eliminated because decoder particles are specifically released from a hybridization sandwich.

15 Figure 2 is a schematic of an assay utilizing strand displacement method, where detection of a single target mRNA is realized on a microfluidic chip. Background is eliminated because decoder particles are specifically released from a hybridization sandwich.

20 Figures 3, 4 and 5 are bar graphs of particle assays showing fluorescence of particles based on varying concentrations of the four fluorescers, 9, 10 diphenylanthracene (DPA), samarium trithenoyltrifluoroacetone bathophenanthroline (SM), europium trithenoyltrifluoroacetone bathophenanthroline (EU), and silicon phthalocyanine.

#### **DESCRIPTION OF THE SPECIFIC EMBODIMENTS**

25 In accordance with the subject invention multiplexed operations may be performed, which require coding for individual events associated with the operation. Where a mixture is to be interrogated for the presence of a plurality of molecules, individually addressable moieties are employed, which are distinguished by the presence of different compositions of fluorescent compounds, where at least one of the fluorescent compounds is a fluorescent lanthanide dye. By providing a spectrum

of fluorescent dye compositions, the individual moieties will have varying fluorescent emission spectra, where the spectrum may be a single wavelength or a plurality of wavelengths, contiguous or non-contiguous, which can be distinguished and related to a entity associated with the moiety. Each spectrum will differ from the spectra of  
5 other addressable moieties at at least one wavelength to be measured. The fluorescent dyes will be associated with a solid moiety, which may be a bulk support, e.g. platen, film, substrate, having the dye compositions at particular sites, or, preferably, particles of from about 5nm to 1 mm diameter, usually in the range of about 10 to about 500 nm, which will provide individual separable sites. By dyeing the individual moieties  
10 with the appropriate fluorescent composition, the different moieties may be distinguished. By coupling the individual moieties with an entity, the emission spectrum will define the nature of the entity.

The subject moieties may be used with various complex samples in a multiplexed format to determine the presence of a plurality of target molecules being  
15 present. Compounds of particular interest include biopolymers, such as polyesters, polyamides and polyethers. The subject moieties include and may be used to detect the presence of different nucleic acid sequences, both naturally occurring and synthetic, including naturally occurring backbones and non-naturally occurring backbones, e.g. phosphates, thiophosphates, phosphoramides, amino acid amides, etc.,  
20 for detecting mutations, alleles, specific DNA genomic sequences, mRNA sequences, cDNA sequences, tRNA sequences, etc. Alternatively, the subject compositions may be used to detect other biopolymer ligands, such as saccharides and poly(amino acids), using poly(amino acids) (proteins or peptides), e.g. lectins, specific binding ligands, e.g. organic molecules, both naturally occurring and synthetic of from about 125 to  
25 5,000Dal. The saccharides may be associated with cellular proteins, toxins, synthetic proteins, oligopeptides from various sources, and the like.

The proteins may be hormones, transcription factors, enzymes, receptors, antibodies, lectins, structural proteins, basement membrane proteins, oncogenic proteins, etc. By having a pair of molecules, which have an enhanced affinity for each  
30 other, e.g. at least  $10^6$  mole<sup>-1</sup>, compared to other molecules which one of the members of the pair is found in admixture, one can obtain binding between the entity and

member of the pair bound to the solid moiety. Employing any of a wide variety of protocols, which have been developed and are described in the literature or future protocols, which may replace such protocols, the formation of the complex may be determined. Once can then identify the particular entity and the other member of the 5 binding pair by the spectrum of the moiety associated with the entity.

The lanthanide dyes comprise the metals Europium (Eu), Samarium (Sm), Terbium (Tb), Dysprosium (Dy), Osmium (Os), and Gadolinium (Gd), which are employed as chelates associated with energy transfer molecules. These energy transfer molecules are squarates, rubrene, phthalocyanines, Nile red, substituted 10 naphthacenes, rhodamine derivatives, oxazines, cyanines, or the like. The energy transfer molecules may be imbibed as separate molecules or may be covalently linked to the metal chelate, preferably covalently linked. Otherwise, to obtain high efficiency of energy transfer, the energy transfer molecules should be used in at least about 3 molar excess. The dyes may be excited with a single excitation light source, 15 particularly at wavelength 337 nm, using a nitrogen ion laser or other convenient excitation light source or a plurality of light sources at selected wavelengths.

In addition to the at least one lanthanide dye, non-lanthanide dyes may be used. Since dyes other than the lanthanide dyes can result in quenching and interfere with emission, usually not more than two non-lanthanide dyes will be used. These 20 dyes will usually be capable of excitation at a wavelength common to the excitation wavelength for the lanthanide dyes and will emit at a wavelength other than the accompanying lanthanide dyes. In some instances, these dyes may serve as a control, serving to normalize the values obtained for the accompanying lanthanide dyes. Illustrative non-lanthanide dyes include 9,10-diphenylanthracene, pyrene, squarene, 25 cyanine, phthalocyanine, etc.

The lanthanide and other dyes may be associated with the solid moiety in a variety of ways: by employing a solution of the dyes whose solvent penetrates or softens the solid moiety; by linking an activated derivative of the dyes to the solid moiety; by combining the metal free chelating agent bound to the solid moiety with 30 the lanthanide metals, or the like. For linking to the solid moiety various functionalities may be employed, such as forming an azo bond with a diazo compound

and an aromatic group, e.g. benzene, an amide bond with an amino group and an acid group, where the acid group may be carboxyl, phosphoryl, sulfonyl, etc., an amino link with a carbonyl and an amine under reductive conditions, an ester group with an alcohol and an acid, ethers, using an alcohol and active halide or equivalent, etc. The 5 chemistry for linking to a solid support is well established and need not be elaborated upon here. The functionality for forming the link may be joined to the chelating agent through a bond or chain of not more than about 36 atoms, usually not more than about 20 atoms, which are usually carbon, nitrogen, oxygen, sulfur and phosphorous, which may include functionalities in the chain as described above.

10 Various entities may be bound to the solid moiety, covalently or non-covalently, either directly or through a linker. The linker may be from 1 to 30 or more atoms in the chain. The linker may provide for release of the particle from the entity, using chemical, electromagnetic, catalytic or thermal means. Various chemically cleavable bonds may be employed, such as disulfides, vicinal glycols, diketones, 15 acetals, o-nitrobenzyl ethers, etc. After performing the determination, the particles may be released from the conjugated entity and then analyzed, particularly where the conjugated entity might interfere with the isolation and identification of the particle. Where a particle is captured by duplex formation between nucleic acids, the particle may be released using denaturing conditions, such as heat, organic solvent, low ionic 20 strength medium, etc.

25 The protocols for which the subject dyed solid moieties may be used will vary widely, but will be associated, for the most part, with determinations involving a plurality of determinations, where one wishes to code for a specific species in a group of like species, such as nucleic acids, including ribonucleic acids, deoxyribonucleic acids, modified nucleic acids, such as nucleoproteins, or other analogs, where the phosphate ester chain has been substituted with a different difunctional moiety from which the bases depend, e.g. amino acid; proteins, which may be involved with binding events, e.g. receptors, antibodies, enzymes, transcription factors, etc., and saccharides. Associated with the nucleic acids, may be the determination of various 30 sequences, the determination of single nucleotide polymorphisms, the determination of transcription as mRNA or cDNA, mutations, chiasmas, inversions, repeats, etc.

Associated with the proteins will be the presence of transcription factors, expression of proteins in relation to cellular status, e.g. neoplastic, stages during meiosis or mitosis, stage of differentiation, response to external stimuli, etc. Saccharides may be involved with detection of unicellular organisms, cellular status, plant cells, etc.

5 For investigating nucleic acids, nucleic acid sequences or their analogs will be linked to the solid moiety, where the sequence will usually having a chain length of at least about 8, more usually at least about 12, and usually not more than about 60, more usually not more than about 36 nucleotides. In some instances, there may be more than an homologous pair of sequences, for example, where bridging amplification is  
10 used. In this instance there will be a 5'-3' sequence homologous with one sequence of a target sequence and a 3'-5' sequence, which will be the same as a sequence in the target sequence spaced apart from the first sequence in the 5'-3' direction. See, for example, U.S. Patent no. 5,641,658. The emission spectrum of the dyes will indicate what the sequences bonded to the solid support are and, to that extent, the target  
15 sequence. Alternatively, one may use the PCR reaction for amplifying sample DNA associated with a specific primer. Other techniques may also be used for amplification, such as cloning, NABSA, SDA, isothermal amplification, etc.

Where one is interested in determining whether there are certain sequences in a sample, for example, for diagnosing the presence of foreign cells in a mammalian sample, the transcription pattern of cells or other interest in the presence of an extended sequence, greater than about 30 nucleotides, the solid moiety can be coded for the sequence, which hybridizes to the target sequence. The sample is combined with the solid moiety under hybridizing conditions, whereby the target DNA will hybridize to an homologous sequence (the hybridizing sequence may differ, usually by  
20 not more than about 10% of the total number of bases, involving insertions, deletions, transitions and transversions), preferably a complementary sequence, under appropriate hybridization conditions, particularly during the wash stage, where non-specific nucleic acid is removed. If desired, the bound sequence could be provided with a photoactivated cross-linking agent, so that after the hybridization, the cross-  
25 linking agent would be photoactivated, and the target sequence would be covalently bonded to the solid moiety.

In order to ascertain the presence of the target sequence bound to the support, there are many protocols, which may be used. If particles are employed, one may label the target sequence with a ligand, so that only those particles to which the target sequence is bound would be captured by the receptor for the ligand. For example, 5 during amplification of a nucleic acid sequence, the primer may be labeled with biotin or other small organic ligand for which a receptor is available. When the target sequence binds to the homologous sequence bound to the dyed particle, the particles to which the target sequence is bound may be sequestered with streptavidin bound to a solid support. The particles may then be individually irradiated and the fluorescence 10 analyzed.

For determining nucleic acids, usually a nucleic acid sample will be processed prior to being used with the particles. Processing may include isolation, purification, fragment formation with restriction endonucleases, amplification with PCR or other technique, denaturation, fusion with primers, attachment to various entities, such as 15 ligands, labels, chelating agents, etc. Depending upon the prior processing, the medium in which the DNA is present may be replaced with a different medium for hybridizing. Once the DNA sample is prepared, it is combined with the particles under hybridizing conditions. Where one is interested in the presence of a sequence, such as identifying an allele, a unicellular organism, e.g. prokaryotic, fungal, protista, 20 etc., mutation, e.g. excision, insertion, transversion or transition, the probe on the particles will be specific for the DNA sequence of interest. By having a ligand on the sample DNA, the duplex of sample DNA and probe will bind to a receptor for the ligand. The receptor may be bound to a surface, such as another particle, e.g. a magnetic particle, a solid support, or other means that allows for sequestering those 25 particles bound to sample DNA from particles that are not bound to sample DNA. One may then isolate the particles and determine their emission spectrum, which will define the probe bound to the particles and identify the DNA sequence.

Where one wishes to reduce the possibility of detecting a homologous sequence as distinct from a complementary sequence, one may use strand 30 displacement, where the ssDNA will bind to the sample DNA releasing the particle. For strand displacement, the target nucleic acid has a ligand for binding to a receptor,

which is bound to a solid support. After capturing the duplexed nucleic acid, one can release particles specifically by adding ssDNA having the desired sequence complementary to the target sequence of interest. This will result in specific release of particles that are bound to the particles, while leaving sequences that are bound and 5 have differences from the target sequence. One may then isolate the individual particles that are released. Again, one will determine the spectrum of the particles to identify the sample DNA.

Instead of determining a sequence, one may be interested in determining single nucleotide polymorphisms (snp's), particularly where the determination is 10 multiplexed. The subject particles allow for high multiplicities of snp determinations in a single reaction vessel. The processed sample would be combined with the particles having bound hybridizing sequences present on the particles, where the emission spectrum of the particles would indicate the sample sequence binding to the particles. In this case, the sample DNA may not be labeled. One would then add 15 probes, which bind to the sample DNA at a site, which upon single nucleotide elongation of the probe, the nucleotide will be complementary to the nucleotide at the putative snp site. By using labeled terminating nucleotide triphosphates, the additional nucleotide will represent whether a snp is present. One may have four reaction vessels, where the particles are associated with snps having the same base or 20 one may have fewer than four reaction vessels, where each terminating nucleotide has a different label for the individual bases. Particularly, one may have different ligands, so that probes bound to sample DNA duplexed to particles would be captured at different sites and be segregated from each other. By releasing the particles and identifying their individual spectra, one would know which sequence had which 25 nucleotide at the putative snp site.

Instead of single nucleotide extension, one could provide for ligation, by having a second primer in solution, where the two primers are separated by the snp. Where the snp is present, the two primers would be ligated and the second primer could allow for selection of those particles where ligation occurred. Other means for 30 providing a moiety to be bound to the particle that allows for selection of the particles may also be employed.

One may use the particles in heterogeneous assays, where the particles serve as labels to identify a plurality of proteins in a sample. For example, when assaying for the status of a cell at various stage of mitosis, in the case of cancer or precancer, cells 5 under stress, or the like, the individual particles would be associated with individual monoclonal antibodies. Various protocols may be used to identify the proteins. The protein fraction of the cells could be spread on a surface to which the proteins would strongly bind. The binding could be specific, where monoclonal antibodies are arrayed on the surface, where the bound monoclonal antibodies bind to an epitope 10 different from the epitope to which the monoclonal antibodies conjugated to the particles binds. One can monitor the activity of cells, using intact cells, either fixed or alive, or cell lysates. By having the particles conjugated with ligands, proteins that bind to surface membrane proteins or antibodies that bind to surface membrane proteins, one can detect the presence of a particular target molecule present on the 15 surface of the cell or internal to the cell. Thus, one can simultaneously monitor the changes in expression in a cell and graph the variations of different proteins simultaneously. By stimulating the cells in various manners, one can obtain a profile of the changes in expression with time as a result of the stimulus.

The particles may be isolated in a variety of ways, using panning, e.g. using 20 pans with dimples and vacuum to pull the particles into individual dimples, fluorescence activated cell sorter, capillary electrophoresis, or the like. Individual particles may then be irradiated and the spectrum analyzed. Generally not more than six different wavelengths will be detectable, usually not more than about four different wavelengths. The spectrum should provide a differentiation between concentrations 25 of at least about 100 RFU, preferably about 200 RFU. Peak heights may be as high as 10,000 RFU or more, frequently not exceeding 5,000 RFU. Time delayed emission maxima may be used for decoding the particle. A commercially available filter wheel may be used in the detection, while a single excitation source is employed, e.g. nitrogen ion laser at 337nm.

30 The particles may be dyed using the appropriate lanthanide dye mixture, the particles and a solvent which able to soften the particles. For particles made of latex

or other similar organic polymer, e.g. poly(methyl methacrylate), polystyrene, polyethylene, polypropylene, poly(vinyl ethyl ether), etc., about 10 to 50 vol.% of ether substituted alkanols boiling above 100°C are used in an aqueous medium at temperatures in the range of about 85 - 95°C. The lanthanide dye mixture is dissolved

5 in the solution and the reaction allowed to proceed for about 1 – 10 minutes, depending upon the volume, concentration of the dyes, nature of the particles and solvent, desired level of dyeing of the particles and the like. The particles may then be isolated, washed with a lower alkanol, e.g. ethanol, and then agitated thoroughly in a milk alkaline solution.

10 In carrying out the multiplexed determinations, usually there will be at least about 5 different moieties employed, more usually at least about 10 different moieties, and not more than about  $5 \times 10^5$ , usually not more than about  $5 \times 10^4$ , moieties. The number of moieties which may be employed will depend upon the nature of the determination, whether the moieties are sites of a solid support or individual particles, 15 sensitivity of discrimination of fluorescence emission, whether one is performing PCR or other process with the particles, the complexity of the sample, and the like. The particles may be magnetic particles or diamagnetic particles. Kits can be provided where from 5 to  $10^6$  or more moieties may be provided, as particles or bulk solid supports.

20 As illustrative of the subject methodology, a microfluidic-based card device designed for a capture-release protocol is employed. The sample is mixed with magnetic particles and introduced into the sample reservoir of the device. The sample members have a ligand label for binding to a receptor. Targets bind to their complementary members on the particles. The contents of the reservoir are then 25 transported by electrokinesis to a magnetic zone, where the magnetic particles are captured and washed free of non-specifically bound components of the sample. The particles are then transported to a site where the receptor bound and particles to which sample is bound are captured. The remaining particles are removed from the site. Where the sample is a nucleic acid sample, with a multiplicity of target sequences, 30 particles may be sequentially removed from the receptor site using strand displacement, by transporting specific nucleic acid strands to the site and incubating,

where the particles having the specific sequence are sequentially released and read or preferably, mixtures of sequences are transported to the site, and groups of particles are released and analyzed individually.

5 The following examples are offered by way of illustration and not by way of limitation.

## EXPERIMENTAL

### Dyeing of Particles with Three Fluorescent Dyes

10

#### MATERIALS

Particles: Seradyn Uniform Microparticles catalog # 1300-2120 (mfg lot # 432 VP2, pkg lot # 1485),

15 2-Ethoxyethanol: Aldrich catalog #25,637-4 (spectrophotometric grade)

Water: Deionized by Millipore's Milli-Q system

9,10- Diphenylanthracene: Aldrich catalog #D20,500-1 (purity 97%)

Samarium trithenoyltrifluoroacetone bathophenanthroline: prepared in-house

Europium trithenoyltrifluoroacetone bathophenanthroline: prepared in-house

20 Ethanol: EM Science, anhydrous (suitable for Histology)

Silicon (IV) phthalocyanine bis (trihexylsilyl oxide) – Aldrich catalog #42,815-9

#### SYNTHESIS

25 To each 10 ml round bottom flask is added 250  $\mu$ l particles in 250  $\mu$ l 2-ethoxyethanol and 500  $\mu$ l water and the particle suspensions are stirred and heated in a 94°C oil bath for 5 minutes.

A mixture of 1 $\mu$ l of 1 mg/ml diphenylanthracene and/or silicon (IV) phthalocyanine bis (trihexylsilyl oxide), Sm (TTA),DPP (the same concentration per assay set;

30 12.5  $\mu$ l, 25  $\mu$ l, 50  $\mu$ l, 100  $\mu$ l of 50 mg/ml) and Eu (TTA),DPP (0, 10, 20, 40, 80, 160  $\mu$ l of 1 mg/ml and 32, 64, 128  $\mu$ l of 10 mg/ml in each assay set) in 2-

ethoxyethanol is added to each particle suspension. The reaction mixtures are heated in a 94°C oil bath for 5 minutes.

The resulting particle suspensions are transferred from the 10 ml round bottom flask to 1.5 ml Eppendorf tubes, then centrifuged at 14,000rpm for approximately 5 minutes.

5 The reaction solvents are drawn off, a 1 ml ethanol wash added and the particle suspensions are sonicated for 30 min.

These sonicated suspensions are centrifuged at 14,000rpm for approximately 5 minutes. The ethanol is drawn off, 1 ml 0.5 N NaOH is added to each tube and 10 the mixtures are again sonicated for 30 min.

## ANALYSIS

15 Each set of particles is assayed using the Hitachi F4600 Fluorometer, with single excitation wavelength of 360 nm. Sample preparation for analysis involves the dilution of 10  $\mu$ l of each particle suspension into 95  $\mu$ l water, then from this dilution 5  $\mu$ l is diluted into 990  $\mu$ l water for analysis. RFU's for DPA, Eu(TTA),DPP, Sm(TTA),DPP and silicon (IV) phthalocyanine bis (triethylsilyl oxide) are recorded at 410.0, 610.0, 645.4 and 690.0 nm emission wavelengths, respectively.

20

## PREPARATION OF IN-HOUSE REAGENTS

## MATERIALS

25 Thenoyltrifluoroacetone: Adrich catalog # T2,700-6 (purity 99%)

Samarium trichloride hexahydrate: Aldrich catalog # 24,880-0 (purity 99+%)

Europium trichloride hexahydrate: Aldrich catalog # 21,288-1 (purity 99.9%)

Bathophenanthroline: Fluka catalog 3 11880 (purity 99+%)

Sodium hydroxide particles: Aldrich catalog # 36,717-6 (20-40 mesh, purity 97%)

30 Ethanol: EM Science, anhydrous (suitable for Histology)

Toluene: Aldrich catalog # 24,451-1 (purity 99.8%)

## SYNTHESIS

5        1. In a 100 ml round bottom flask was dissolved 2.00 gm thenoyltrifluoroacetone  
          (9.00 mmoles, 3 equiv.) in 15 ml ethanol.

2. 0.36 gm sodium hydroxide particles (9.00 mmoles, 3 equiv.) were added and the  
reaction mixture was stirred and heated at 40°C for 30 minutes.

3. To the reaction mixture was added 1.09 gm samarium (or europium) trichloride  
10      hexahydrate (3.00 mmoles, 1 equiv.) and stirring and heating of the reaction  
mixture was continued for one hour.

4. First the solvent was removed in vacuo, then sodium chloride was removed by  
dissolving the entire solid in hot toluene and filtration.

5. To the filtrate was added 1.0 gm bathophenanthroline (3.00 mmoles, 1 equiv.) and  
15      the reaction mixture was run on a rotary evaporator for 1½ hours, replenishing the  
toluene throughout the reaction time and removing it at the end of the reaction  
time.

Europium trithenoyltrifluoroacetone bathophenanthroline [Eu (TTA),DPP]: same  
20      procedure as above, substituting europium trichloride hexahydrate for samarium  
trichloride hexahydrate.

## PARTICLE DYEING REAGENT CHART

ASSAY # (BEAD #)	VOLUME OF DPA (ul)	VOLUME OF Sm (TTA)3DPP	CONCENTRATION OF Eu(TTA)3TPP (ul)	VOLUME OF Eu(TTA)3TPP (ul)	VOLUME OF 2-Ethoxyethanol
1A	1	50	1	0	195
1B	1	50	1	10	185
1C	1	50	1	20	175
1D	1	50	1	40	155
1E	1	50	1	80	115
1G	1	50	10	32	183
1H	1	50	10	64	131
1I	1	50	10	128	67
3A	1	12.5	1	0	232.5
3B	1	12.5	1	10	222.5
3C	1	12.5	1	20	212.5
3D	1	12.5	1	40	192.5
3E	1	12.5	1	80	152.5
3F	1	12.5	1	160	72.5
3G	1	12.5	10	32	220.5
3H	1	12.5	10	64	168.5
3I	1	12.5	10	128	104.5
4A	1	100	1	0	145
4B	1	100	1	10	135
4C	1	100	1	20	125
4D	1	100	1	40	105
4E	1	100	1	80	65
4F	1	100	10	16	129
4H	1	100	10	64	64
4I	1	100	10	128	128

It is evident from the above results and the accompanying figures that the subject particles allow for multiplexing various determinations and operations, where one can distinguish events occurring in relation to the particles by identifying the emission spectrum of the particle. Of course only individual points need be determined and by using the isobestic point for two or more combinations of dyes, one has a value to normalize the other values.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

DRAFT IN PROGRESS

## WHAT IS CLAIMED IS:

1. In a method for performing a multiplexed determination on a surface of a solid moiety comprising independently addressable sites, the improvement which comprises:

employing mixtures of lanthanide dyes for encoding said moieties in said solid moiety; and

decoding said sites by their emission spectra.

2. A method according to Claim 1, wherein said lanthanide dyes are chelates of Eu, Sm, Tb, and Dy.

3. A method according to Claim 1, wherein said lanthanide dyes code for a nucleic acid sequence bound to said moieties.

4. A method according to Claim 3, wherein said lanthanide dyes are chelates of Eu, Sm, Tb, and Dy

5. A method according to Claim 1, wherein said moieties comprise from 0 to 2 fluorescent dyes other than lanthanide dyes having an absorption wavelength overlapping the absorption wavelength of at least one of said lanthanide dyes.

6. A method according to Claim 1, wherein said moieties are particles of an average diameter in the range of about 5 nm to 1 mm.

7. A method for determining the presence of at least 5 different nucleic acid sequences in a sample comprising a mixture of nucleic acid molecules, said method comprising:

combining independently addressable particles with said sample, wherein said independently addressable particles are characterized by comprising a dye composition consisting of at least one lanthanide dye and from 0 to 2 fluorescent dyes other than lanthanide dyes, having an absorption wavelength overlapping the absorption wavelength of at least one of said lanthanide dyes, having nucleic acid

oligomers bound to the surface of said particles, wherein the sequence of said oligomers bound to said particle is related

to the emission spectrum of said dye composition of said particle under hybridization conditions;

separating particles to which sample nucleic acid molecules have hybridized; and

identifying the sequence of said nucleic acid molecules bound to said particles by the emission spectra of said particles.

8. A method according to Claim 7, including the additional step of amplifying nucleic acid molecules or portions thereof in said sample.
9. A method for identifying at least 5 single nucleotide polymorphisms in a sample comprising a mixture of nucleic acid molecules, said method comprising:

combining independently addressable particles with said sample, wherein said independently addressable particles are characterized by comprising a dye composition consisting of at least one lanthanide dye and from 0 to 2 fluorescent dyes other than lanthanide dyes, having an absorption wavelength overlapping the absorption wavelength of at least one of said lanthanide dyes, having nucleic acid oligomers bound to the surface of said particles, wherein said oligomers bound to each particle hybridize to a nucleic acid molecule comprising a single nucleotide polymorphism;

extending said oligomers with a template dependent DNA polymerase, wherein extension requires the presence of said single nucleotide polymorphism;

separating particles having extended oligomers; and

determining the single nucleotide polymorphisms by determining the emission spectrum of each of said particles.

10. A kit comprising a plurality of independently addressable solid surfaces,

wherein said independently addressable particles are characterized by comprising a dye composition consisting of at least one lanthanide dye and from 0 to 2 fluorescent dyes

other than lanthanide dyes, having an absorption wavelength overlapping the absorption wavelength of at least one of said lanthanide dyes.

11. A kit according to Claim 10, having at least 10 different independently addressable solid surfaces.

12. A kit according to Claim 11, wherein said independently addressable solid surfaces are particles.

13. A kit according to Claim 10, wherein said lanthanide dyes are chelates of Eu, Sm, Tb, and Dy.

14. A kit according to Claim 13, having from 1 to 2 fluorescent dyes other than lanthanide dyes, having an absorption wavelength overlapping the absorption wavelength of at least one of said lanthanide dyes.

15. A kit according to Claim 10, wherein to each of said independently addressable surfaces is bound a different DNA oligomer, wherein the emission spectrum of each surface defines the sequence of the oligomer bound to said surface.

16. A kit according to Claim 15, wherein said surfaces are particles.

17. A kit according to Claim 10, wherein to each of said independently addressable surfaces is bound a poly(amino acid), wherein the emission spectrum of each surface defines the sequence of poly(amino acid) bound to said surface.

18. A kit according to Claim 17, wherein said surfaces are particles.

# INDIVIDUALLY ADDRESSABLE SOLID SURFACES FOR MULTIPLEXED OPERATIONS

## ABSTRACT OF THE DISCLOSURE

Solid moieties are provided which are individually addressable by using individual or combinations of dyes, including at least one lanthanide dye. The solid moieties may be a bulk solid having individual sites or particles, where each site can provide a unique emission spectrum defining an entity bound to the site. The individually addressable moieties may be used in multiplexed determinations for determining the presence of biopolymers or other mixture of differentiable entities. Different protocols are provided for the determinations.

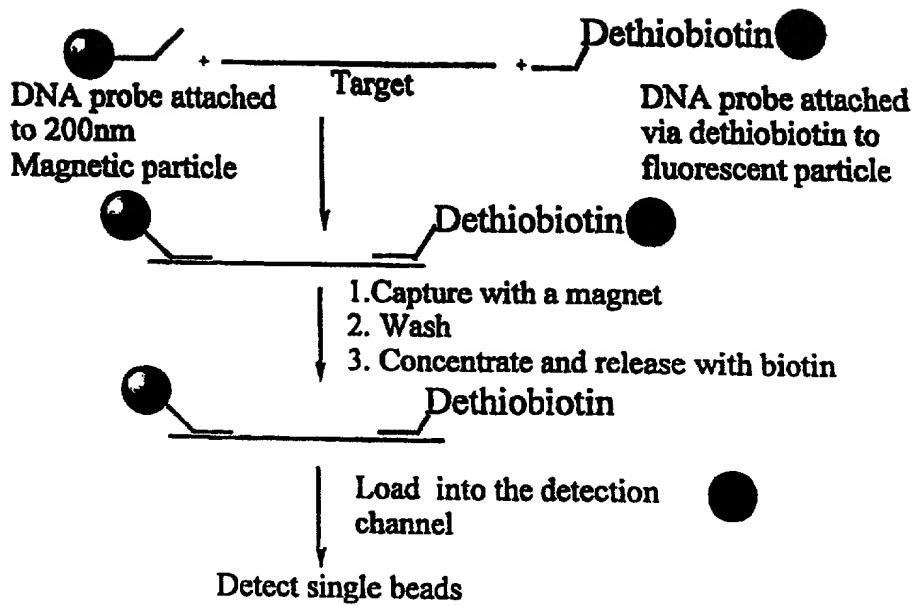


FIGURE 1

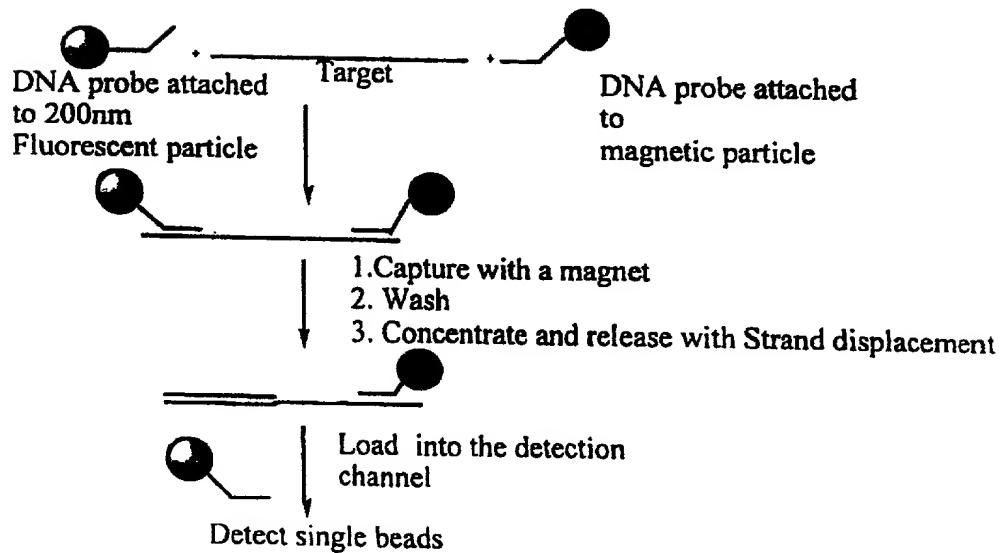


FIGURE 2

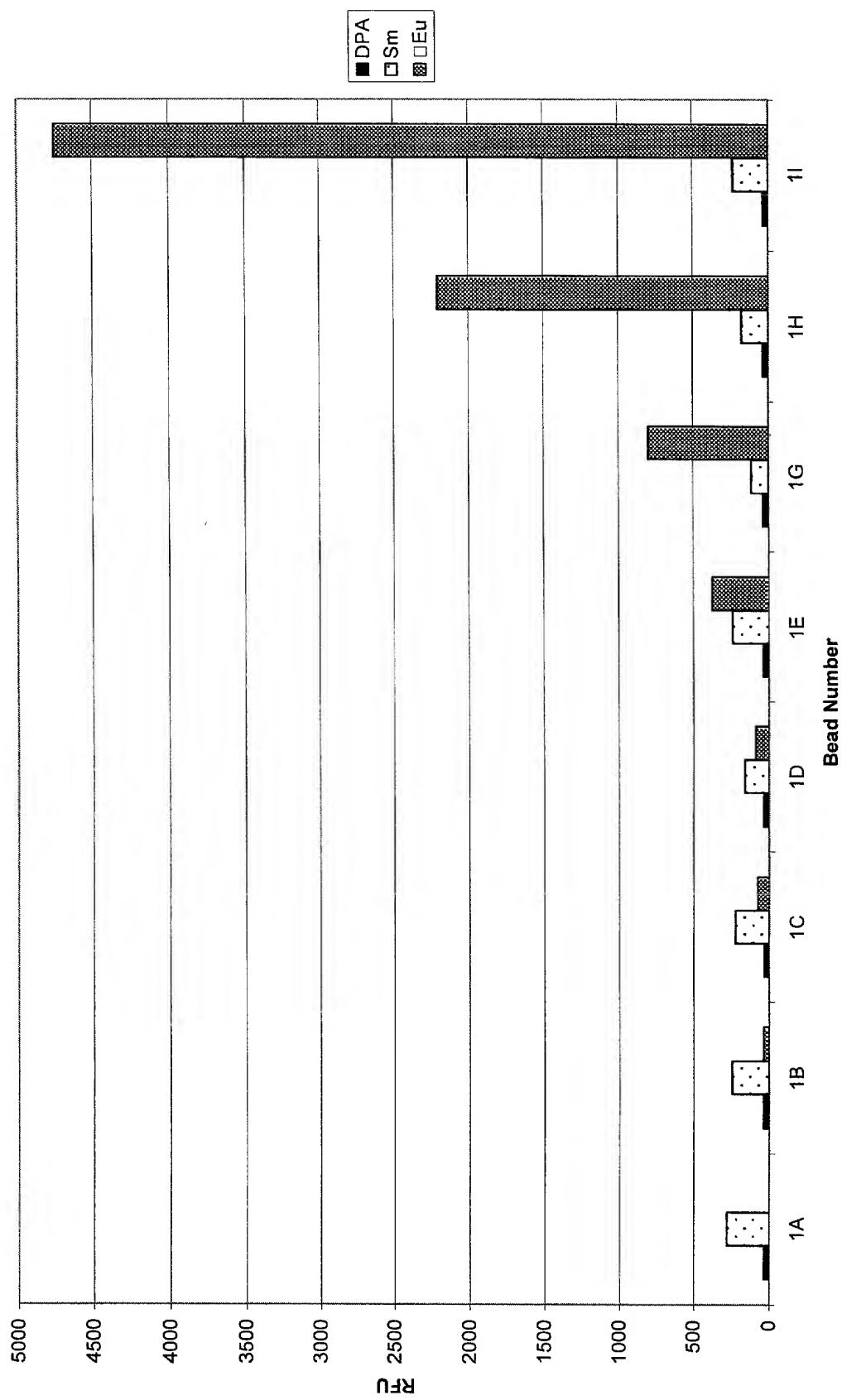


FIGURE 3

SHEET 2 OF 4

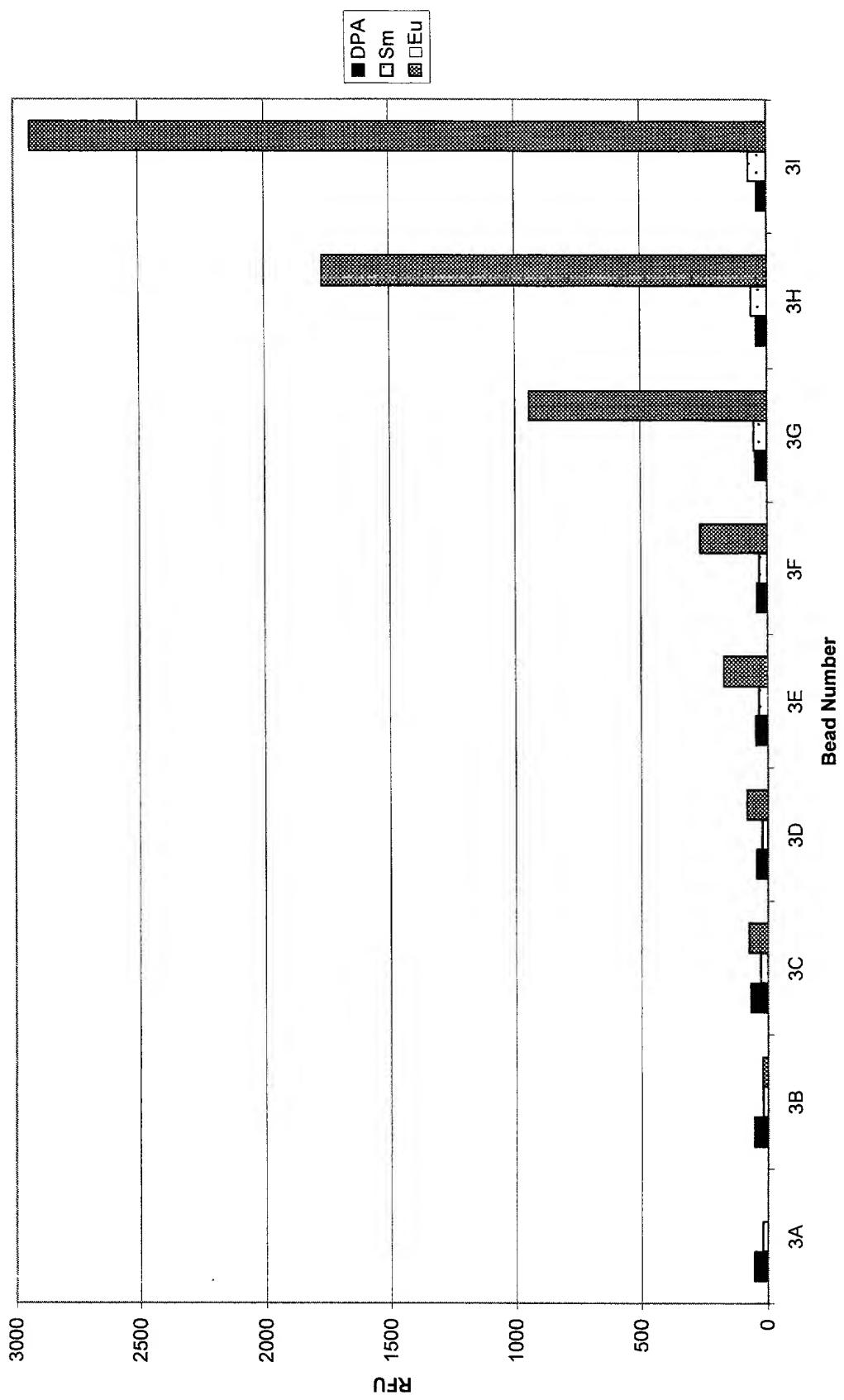


FIGURE 4

SHEET 3 OF 4

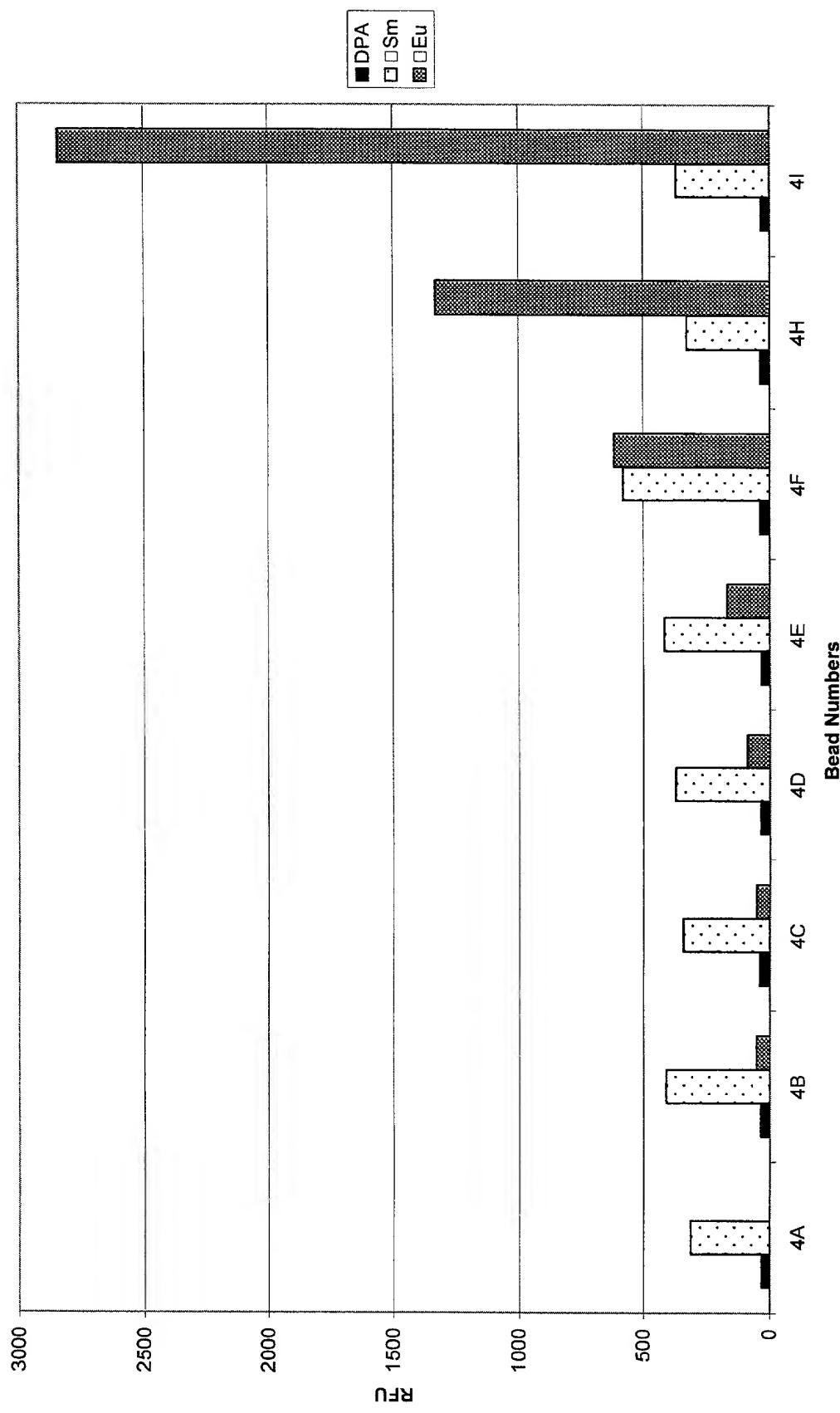


FIGURE 5

SHEET 4 OF 4

**COMBINED INVENTOR  
DECLARATION AND POWER OF ATTORNEY**

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**Individually Addressable Surfaces For Multiplexed Operations**

the specification of which

(check one)  Is attached hereto.  
 Was filed on \_\_\_\_\_ and has been assigned Serial Number \_\_\_\_\_  
 Was filed on December 15, 1999 as Attorney Docket No. ACBI.031.01US.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known to me to be material to patentability of this application as defined in Title 37, Code of Federal Regulations, §1.56 and, if applicable, all such information under 37 CFR § 1.56 which became available between the national or PCT International filing date of the prior application and the filing date of this application.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

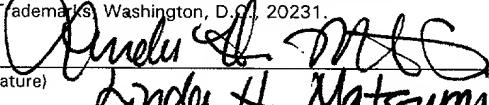
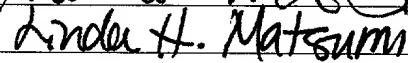
Priority Claimed

(number)	(Country)	(Day/Month/Year Filed)	[ ]	[ ]
			Yes	No

"Express Mail" Label No.: EL398977959US

Date of Deposit: 15 Dec (99)

I hereby certify under 37 C.F.R. 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

  
(Signature)  
  
(Printed Name)

Prior Foreign Application(s) Priority Claimed

(number) (Country) (Day/Month/Year Filed)  Yes  No

Prior Foreign Application(s) Priority Claimed

(number) (Country) (Day/Month/Year Filed)  Yes  No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC §112 I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56, which became available between the filing date of the prior application and the national or PCT international filing date of this application:

60/113,853 December 24, 1998 Pending  
(Application Serial No.) (Filing Date) (Status)

(Application Serial No.) (Filing Date) (Status)

(Application Serial No.) (Filing Date) (Status)  
\* designating the U.S.

I hereby appoint:

BARBARA RAE-VENTER, Ph.D., Reg. No. 32,750  
BERTRAM I. ROWLAND, Ph.D., Reg. No. 20,015

as my attorneys or agents with full power of substitution and revocation to prosecute my above-identified application for Letters Patent and to transact all business in the Patent Office connected therewith.

Direct all telephone calls to Barbara Rae-Venter, Ph.D. at (650) 328-4400.

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Palo Alto, California 94306-0039

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Full name of third joint inventor, if any:

Inventor's signature: \_\_\_\_\_

Date: \_\_\_\_\_

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